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Combined First and Second
Interim Technical Reports
(Subline Items 0001AA and 0001AB)

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Title Proposed by Firm: "Human red blood cell freezing with and without metabolizable cryopreservatives, molecular distillation drying, storage, and subsequent rehydration."

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Abstract

Studies to determine the optimum choice of freezing and drying methods for fresh human erythrocytes have demonstrated that cryoprotection and anhydrous stabilization with 10-12% (w/v) combinations of sucrose and raffinose prior to slow-rate cooling at -5°C/min, followed by molecular distillation drying at rates over a relatively broad range, with subsequent rehydration in liquid medium at 20-37°C, result in the best structural preservation and minimum cell fragmentation or clumping.

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Introduction

This report represents the results of two-thirds of the work to be undertaken under this Phase I grant. Much progress has been made toward reaching our goal of optimizing the freezing and drying process for erythrocyte preservation. One important finding was that slow cooling with limited amounts (7.5%) of non-toxic cryoprotectants (sucrose) appear to be sufficient to allow the freezing and drying of erythrocytes with minimum deleterious effects as seen by light microscopy.

Experimental Series 1

Determine the Criteria Required to Achieve Complete Vitrification of the Sample

The purpose of this series of experiments was to choose an appropriate method to cryopreserve erythrocytes preparatory to molecular distillation drying. We believed that it might be necessary to vitrify the water in the erythrocytes by ultrarapid cryofixation or by liquid nitrogen plunge after use of high-dose cryoprotectants. We have found that neither technique is necessary for the outcome we desire. This is fortunate, since our experiments have shown that ultrarapid cryofixation yields cell clumping, membrane fragmentation, and cell loss, while rapid cooling in liquid nitrogen requires excessive cryoprotection, which is what these studies are designed to overcome.

In Table 1, we list the post-freezing/thawing cell counts after using a multitude of cryoprotectants at two dosages and two periods of incubation. The counts were made after slow-rate cooling and rewarming at 37°C. Most of the slow-rate cooled erythrocytes in cryoprotectants looked good under phase contrast microscopy (except hydroxyethyl starch, which showed clumping and fragmentation and is not included in the table). Relatively good preservation was incurred in the case of liquid nitrogen plunged cells after low-dose, non-toxic cryoprotection, but cell counts were not technically possible, rather we obtained photographs of each experiment which demonstrated the retention of structure and morphology. Liquid nitrogen plunge was inferior to cooling at -5°C/min. as judged by phase contrast microscopy.

The red blood cell samples cooled in a Planar controlled-rate freezer at a rate of -5° C/min, yielded about 30,000 recoverable cells in 7.5% DMSO, glycerol, glucose, propylene glycol or sucrose after 30 minutes incubation, and only 1,000 - 6,000 cells after 5 minutes incubation. However, this difference was not seen with other cryoprotective agents, like proline, raffinose, dextran, and PVP. This might mean that the first set of agents is rapidly penetrating, while the second set is not. Sucrose falls in the first set and raffinose in the second. It is interesting that in the experimental results presented later in this report, sucrose and raffinose will ultimately yield the best results. Using 15% amounts of cryoprotectant agents resulted overall in a decrease rather than an increase in cell number at 30 minutes. These results would indicate a cytotoxicity effect at that concentration and time duration.

Table 1.

Erythrocyte counts after cooling at -5° C/min (slow-rate cooling) in the listed cryoprotectant and then rewarming at 37° C.

Cryoprotectant	Concentration (%)	Time (min)	No. of Cells
Non-Frozen Control			38,025
Frozen Control (PBS)			2,574
Dimethylsulfoxide (DMSO)	7.5 (v/v)	5	6,585
DMSO	15 (v/v)	5	1,605
DMSO	7.5 (v/v)	30	28,807
DMSO	15 (v/v)	30	2,468
Glycerol	7.5 (v/v)	5	4,195
Glycerol	15 (v/v)	5	6,217
Glycerol	7.5 (v/v)	30	29,981
Glycerol	15 (v/v)	30	1,148
Glucose	7.5 (w/v)	5	1,042
Glucose	15 (w/v)	5	5,187
Glucose	7.5 (w/v)	30	35,136
Glucose	15 (w/v)	30	2,367
Propylene Glycol	7.5 (v/v)	5	1,695
Propylene Glycol	15 (v/v)	5	999
Propylene Glycol	7.5 (v/v)	30	30,739
Propylene Glycol	15 (v/v)	30	595

(continued)

Table 1 (Continued)

Cryoprotectant	Concentration (%)	Time (min)	No. of Cells
Sucrose	7.5 (w/v)	5	3,760
Sucrose	15 (w/v)	5	3,818
Sucrose	7.5 (w/v)	30	32,746
Sucrose	15 (w/v)	30	3,149
Dextran	7.5 (w/v)	5	1,561
Dextran	15 (w/v)	5	1,972
Dextran	7.5 (w/v)	30	645
Dextran	15 (w/v)	30	1,075
Proline	7.5 (w/v)	5	5,238
Proline	15 (w/v)	5	4,332
Proline	7.5 (w/v)	30	5,204
Proline	15 (w/v)	30	6,017
Raffinose	7.5 (w/v)	5	2,779
Raffinose	15 (w/v)	5	6,790
Raffinose	7.5 (w/v)	30	2,187
Raffinose	15 (w/v)	30	3,662
Butanediol	7.5 (v/v)	5	3,297
Butanediol	15 (v/v)	5	8,415
Butanediol	7.5 (v/v)	30	6,216
Butanediol	15 (v/v)	30	5,172
Polyvinyl pyrrolidone (PVP)	7.5 (w/v)	5	11,793
PVP	15 (w/v)	5	9,931
PVP	7.5 (w/v)	30	11,074
PVP	15 (w/v)	30	8,862

Experimental Series 2

Determine Criteria for Optimum Drying

Experiments in this series were undertaken to determine whether optimum cryoprotectants and cooling rates for liquid nitrogen storage and thawing are compatible with molecular distillation drying.

We began by employing our original molecular distillation drying cycle, specified in Table 2. This cycle was developed on the basis of optimized structural preservation of cryofixed cells and tissues as determined by transmission electron microscopy. We also tested a shortened cycle specified in Table 3. In the case of both of these drying cycles, the erythrocytes were

Table 2. Original Drying Cycle

Temperature Range	Programmed Temperature Change
-190° C to -140° C	+10° C/hr
-140° C to -70° C	+1° C/hr
-70° C to +20° C	+10° C/hr

Table 3. Shortened Drying Cycle

Temperature Range	Programmed Temperature Change
-190° C to -135° C	+10° C/hr
-135° C to -110° C	+1° C/hr
-110° C to +20° C	+10° C/hr

prepared by the following cooling rates: ultrarapid cryofixation, liquid nitrogen plunge, and programmed controlled-rate cooling at -20° C/min. -5° C/min., and -1° C/min. The cryoprotectants used were different concentrations (10 m<u>M</u>-1 <u>M</u>) of sucrose and raffinose, and combinations thereof, with the anhydrous dry protectant trehalose in PBS. The cells were dried, unloaded under dry nitrogen and rehydrated in phosphate buffered saline. The cells were visualized by light microscopy. The results demonstrated differences among the freezing techniques, but not between the original drying cycle and the shortened drying cycle. Specifically, the best result was obtained by using controlled cooling rates of -5° C/min and -1° C/min., using sucrose, raffinose and combinations thereof, between 3% and 12% (w/v) of each sugar. It was found that high concentrations near 10% - 12% of each with cooling at -5° C/min was best. The other cooling techniques were less effective, as shown in Table 4.

Table 4. Effectiveness of cooling rate and molecular distillation drying cycle on erythrocytes protected with 10 m<u>M</u> sucrose and 10 m<u>M</u> trehalose. Evaluation was based on morphology and integrity as determined by phase contrast microscopy (0=worst,+++=best)

	MDD Cycle	
Cooling Method/Rate	Orig.	Shortened
-1° C/min (controlled rate)	+++	+++
-5° C/min (controlled rate)	+++	+++
-20° C/min (controlled rate)	++	++
-700° C/sec (LN_2 plunge)	+	+
-100,000° C/sec (ultrarapid cryofixation)	0	0

Cryoprotection and dry protection of the erythrocyte was studied by using electroporation. Partial success has been obtained in that we are getting about 1% of the cryo- or dry-protectants to enter the erythrocytes. In Phase II, we will be exploring this technique to improve the method, including greater uptake and post-uptake resealing of the membrane.

In determining the characteristics of the drying cycle, it is necessary to assess whether the addition of cryoprotective agents causes a change in the thermal characteristics of the phase transitions occurring in the cryoprepared sample. To investigate this we have conducted differential scanning calorimetry analyses of sucrose and raffinose samples in comparison to DMSO, a more widely used cryoprotective agent. Data shown in Table 5, together with printouts included in Appendix 1, indicate a transition window (Tg to Tc) in the range -148° C to -117° C largely unaffected by the added cryoprotectant agent and not dramatically different from published data of the Tg and Tc for vitrified water. All samples used in this comparative analysis were ultrarapidly cooled to produce single phase (vitreous or near vitreous) samples in order to simplify analysis.

Table 5. Tg and Tc values of vitrified erythrocytes in each of three cryoprotectants as determined by differential scanning calorimetry.

Sample No.	Cryoprotectant	Conc.(v/v)	Tg(°C)	Tc(°C)
1	DMSO	7.5%	-148	ND
2	DMSO	7.5%	-143	-117
3	DMSO	7.5%	-139	-120
4	Sucrose	7.5%	-123	-118
5	Sucrose	7.5%	-147	ND
6	Raffinose	7.5%	-148	-121
7	Raffinose	7.5%	-147	-117

The transitions are relatively wide. In all of them, the Tg is between -135° and -147°C and the Tc is around -117°C to -125°C. This data has caused us to devise a new drying cycle specified in Table 6.

Table 6. Drying Cycle Based on Tg and Tc Values

Temperature Range	Programmed Temperature Change
-190°C to -155°C	+10°C/hr
-155°C to -110°C	+1°C/hr
-110°C to +20°C	+10°C/hr

Experimental Series 3

Determine Optimum Criteria for Rehydration

The purpose of these experiments were to determine whether improved recovery after drying could be obtained by various media (Eagle's MEM, Ham's F-12, RPMI/1640), additives (7.5% w/v sucrose, 2 mM ATP), rehydrating in vapor or liquid phase, and the rehydration temperature (4°C, 20°C, 37°C). Assessment of the results was by light microscopy.

Both the use of ATP and the use of vapor rehydration yielded clumps and fragments of erythrocytes and were deemed to be unusable. Liquid rehydration at 20° C or 37° C is superior to 4° C. The results of the combinations of cryoprotectants and rehydration media are listed in Table 7. Finally, the use of 7.5% w/v sucrose in the rehydration media did not improve the results.

Table 7. Conjoint effect of choice of cryoprotectant and choice of rehydration media on erythrocyte morphology and integrity determined by light microscopy.

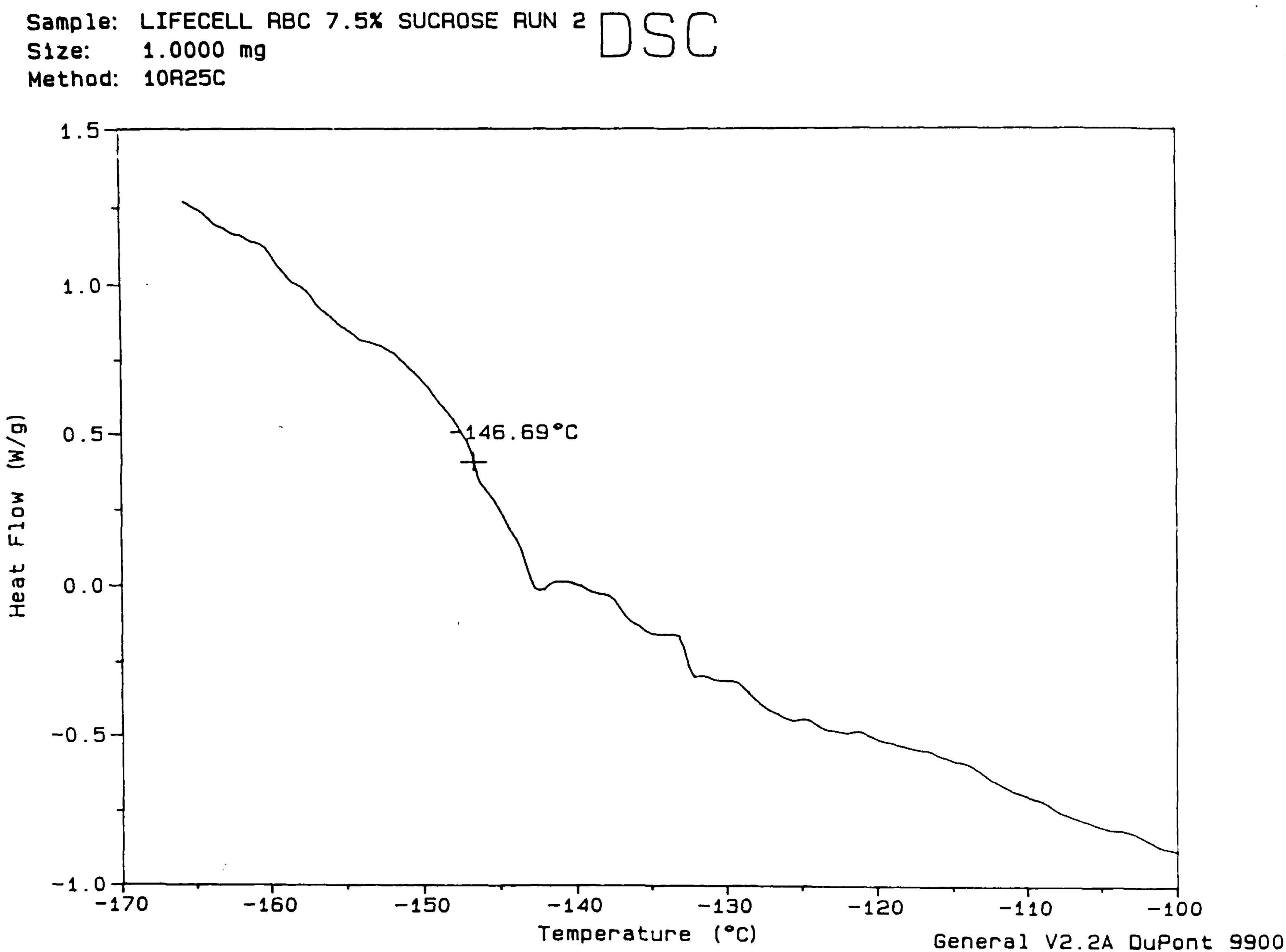
	Rehydration Media			
Cryoprotectant (7.5% v/v)	PBS	Eagle's MEM	Ham's F-12	RPMI 1640
Sucrose	+++	++	+++	+
Raffinose	+++	++	++	+
DMSO	0	0	0	0

Experimental Series 4

Determine if Prestressing the Cells Increases Their Ability to Survive the Cryoprotection and Molecular Distillation Drying Process

In general, we have become more pessimistic that prestressing would help prepare an erythrocyte for freezing and drying, so we have truncated these experiments. Nevertheless, we have temperature-stressed erythrocytes for up to 48 hours at 4° C and have not been able to detect any changes in their ability to withstand freezing, drying, and rehydration by the various methods. Our conclusion is that for a non-nucleated end cell such as the erythrocyte, prestressing investigations will be of little benefit to the final outcome.

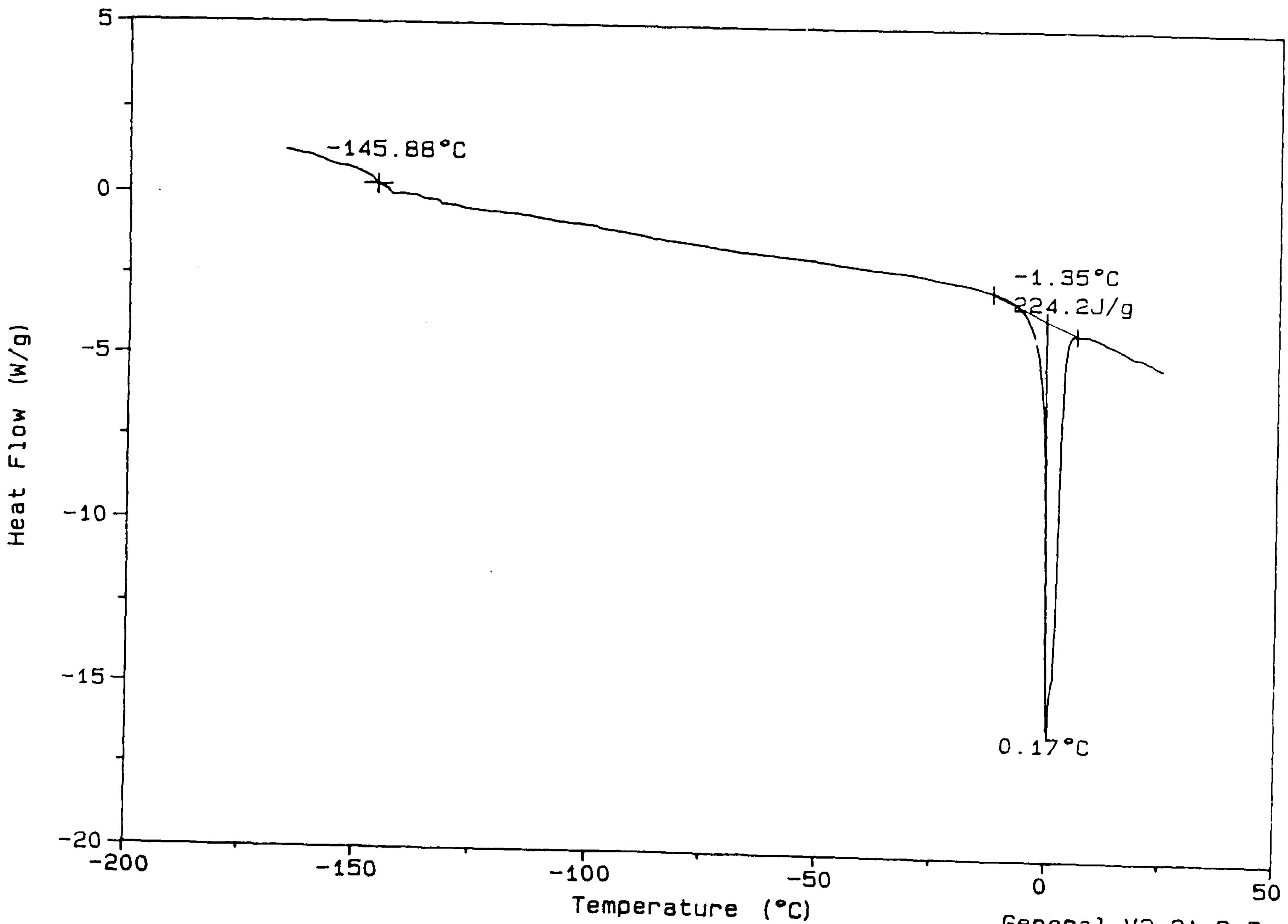
Appendix 1



Sample: LIFECELL RBC 7.5% SUCROSE RUN 2
Size: 1.0000 mg
Method: 10R25C
DSC
-146.69°C
Heat Flow (W/g)
1.5
1.0
0.5
0.0
-0.5
-1.0
-170
-160
-150
-140
-130
-120
-110
-100
Temperature (°C)
General V2.2A DuPont 9900

Sample: LIFECELL RBC 7.5% SUCROSE RUN 2
Size: 1.0000 mg
Method: 10R25C

DSC



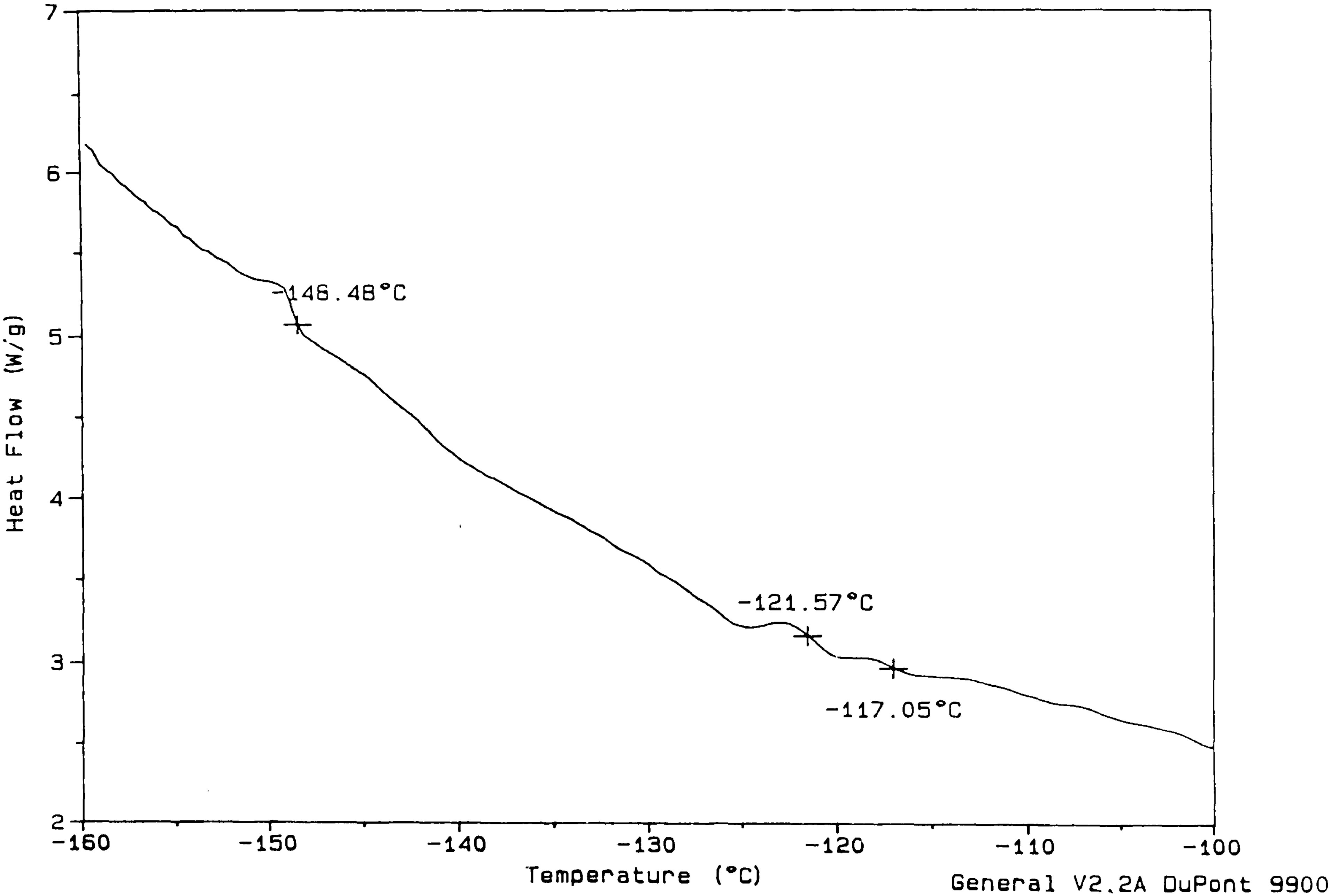
General V2.2A DuPont 9900

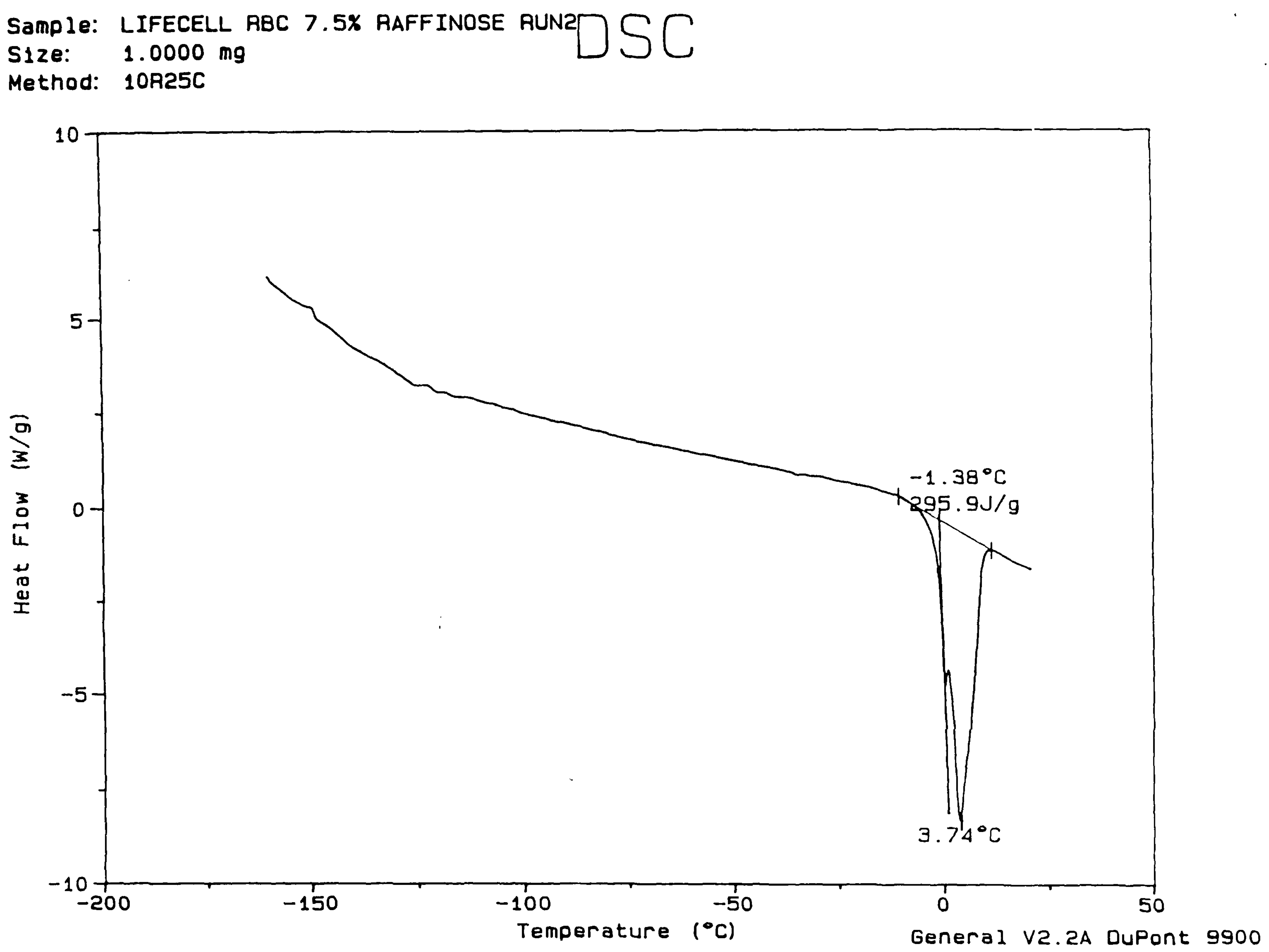
Sample: LIFECELL RBC 7.5% RAFFINOSE RUN2

Size: 1.0000 mg

Method: 10R25C

DSC

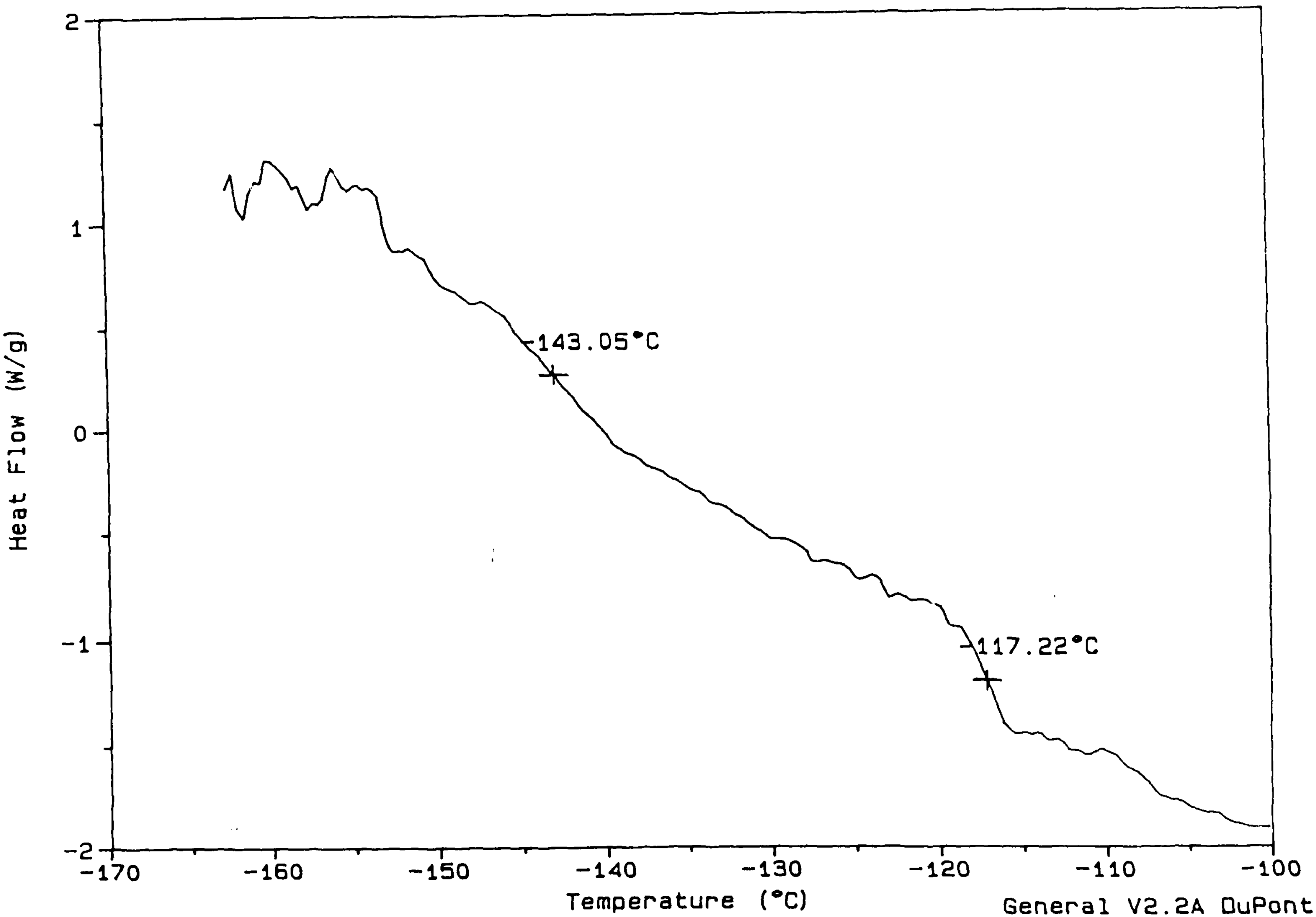




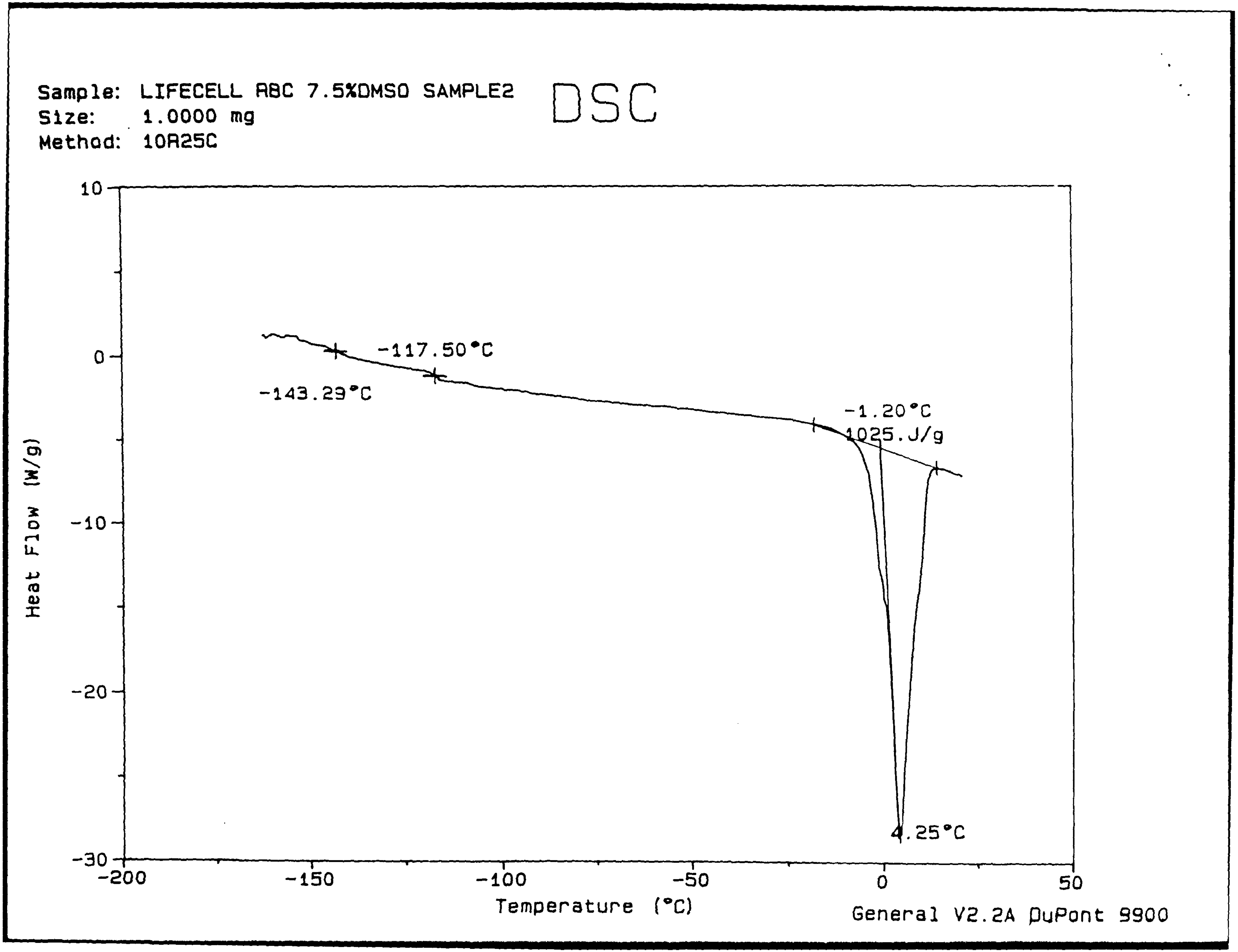
Sample: LIFECELL RBC 7.5% RAFFINOSE RUN2
Size: 1.0000 mg
Method: 10R25C
DSC
10
5
0
-5
-10
Heat Flow (W/g)
-1.38°C
295.9J/g
3.74°C
-200
-150
-100
-50
0
50
Temperature (°C)
General V2.2A DuPont 9900

Sample: LIFECELL RBC 7.5%DMSO SAMPLE2
Size: 1.0000 mg
Method: 10R25C

DSC



General V2.2A DuPont 9900


Sample: LIFECELL RBC 7.5%DMSO SAMPLE2
Size: 1.0000 mg
Method: 10R25C
DSC
-117.50°C
-143.29°C
-1.20°C
1025.J/g
4.25°C
Heat Flow (W/g)
10
0
-10
-20
-30
-200
-150
-100
-50
0
50
Temperature (°C)
General V2.2A DuPont 9900